Response of *Staphylococcus aureus* Isolates from Bovine Mastitis to Exogenous Iron Sources¹

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ABSTRACT

Staphylococcus aureus can survive in conditions of extremely low iron concentration. The ability of *S. aureus* to use two exogenous hydroxamate types of siderophores (desferrioxamine and ferrichrome) and four iron-containing proteins found in cattle (hemin, hemoglobin, ferritin, and lactoferrin) were tested on 16 reference and clinical isolates. For all strains tested, ferrichrome and desferrioxamine showed strong growth-promoting activities in a disk diffusion assay and in liquid medium. The heme proteins hemin and hemoglobin were also found to support growth in culture media lacking other iron sources, while lactoferrin failed to do so. On media containing the iron chelator dipyrindyl, ferritin induced a growth inhibition effect that was further enhanced in the presence of lactoferrin in seven of the 13 tested strains. *Staphylococcus aureus* was able to bind hemin and the level of binding activity was not increased after growth in iron-rich or -poor media. Dot-blot competition tests showed that biotin-labeled lactoferrin binds to *S. aureus*, and this binding can be inhibited by unlabeled lactoferrin. Expression of lactoferrin-binding activity was independent of the level of iron in the medium and the iron saturation status of lactoferrin. For each strain tested, ligand blots showed lactoferrin-binding proteins of molecular weights ranging from 32 to 92 kDa. Possible functions of these lactoferrin-binding proteins could not be related to iron acquisition mechanism in *S. aureus*.

(**Key words:** *Staphylococcus aureus*, iron deprivation, mastitis, siderophore)

Abbreviation key: streptavidin-AP = streptavidin-alkaline phosphatase, CAS = chrome azurol S, DFO = desferrioxamine B, DMSO = *N,N*-dimethylsulfoxide, EDDHA = ethylenediamine di-O-hydroxyphenyl-

cetic acid, MHA or MHB = Mueller Hinton agar plate or broth, NBT/BCIP = nitro blue tetrazolium/5 bromo-4-chloro-3-indolyl phosphate toluidine, NHS-biotin = N-hydroxy-succinimide-biotin, TSB = Tris-saline buffer.

INTRODUCTION

Staphylococcus aureus is an important human and animal pathogen. This bacterium is one of the most common causes of bovine mastitis, which is a disease responsible for important economic losses in dairy production (National Mastitis Council, 2000). Despite the progress in antimicrobial therapy, the treatment and prevention of *S. aureus* infections remain serious clinical problems. The determination of the mechanisms of virulence of *S. aureus* might enable the development of new approaches to fight this bacterium. Several secreted products, including toxins and hemolysins have already been identified as virulence factors (Novick, 1990; Trivier and Courcol, 1996). However, it is likely that several other virulence factors are essential for this bacterium to cause mastitis.

Pathogenic bacteria have a strict nutritional requirement for iron. In vivo, they must contend with the natural ability of the host to withhold free iron by binding it in the form of iron-protein complexes such as transferrin, lactoferrin, hemoglobin, and ferritin (Aisen and Leiman, 1972; Litwin and Calderwood, 1993). Competition for iron between a host and bacteria is an important factor determining the course of a bacterial infection. Siderophores are synthesized by many microorganisms grown under iron-limiting conditions. These are molecules of low molecular weight capable of chelating iron with high affinity. They scavenge iron in the extracellular environment, and the iron complexes formed by these molecules are retrieved and actively taken up by specific transport systems present on the bacterial cell surface (Griffiths, 1987). Iron is then released from the ferric siderophore complex within the cell by an enzymatic system (Hider, 1984; Neilands and Nakamura, 1991). Considerable structural variation exists among siderophores;

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however, they are generally grouped according to the chemical group that is involved in the binding of iron (Neilands and Nakamura, 1991). The ability to produce and utilize siderophores has been frequently linked to the virulence of certain pathogenic bacteria (Martinez, et al., 1990). In addition, restricted availability of iron by host functions is an important signal leading to the enhanced expression of a wide variety of bacterial toxins and other virulence determinants.

Little is known about the iron-acquisition mechanisms of *S. aureus*. Under iron-restricted growth conditions, *S. aureus* secretes two carboxylate-type siderophores (staphyloferrin A and B) and a chemically uncharacterized siderophore named aureochelin (Haag et al., 1994; Modum et al., 2000). Trivier and Courcol (1996) have reported that *S. aureus* isolated from humans can survive in an iron-restricted environment by using transferrin, lactoferrin, hemoglobin, and ferroxamines as sources of iron. Analysis of the response to cell surface antigens during *S. aureus* infection has allowed the identification of a cell surface transferring-binding protein of 42 kDa (Modum et al., 2000). Intracellular iron can be stored on a protein called ferritin (Ghio et al., 1998), which might be a significant source of iron for intracellular bacteria.

The aim of the present study was to investigate the capacity of *S. aureus* strains isolated from bovine mastitis cases to obtain iron from exogenous hydroxamate siderophores and some host iron-containing proteins. We report that *S. aureus* can use several of those compounds for growth and that this microorganism has a high affinity iron-uptake system.

MATERIALS AND METHODS

Reagents

The hydroxamate siderophores ferrichrome A and desferrioxamine B (**DFO**) were supplied by Sigma Chemicals (St. Louis, MO). The iron-chelating portion of ferrichrome contained the tripeptide sequence *N*⁵-acetyl-*N*⁵-hydroxy- L-ornithine and the 1-amino- ω -hydroxylamino alkanes coupled by succinates for DFO. Bovine lactoferrin was from Besnier (San Juan Capistrano, CA) while bovine hemin, hemoglobin and horse spleen ferritin were from Sigma. Lactoferrin, hemoglobin, and ferritin were stored at -20°C at a concentration of 20 to 100 mg/ml in water. Siderophores and hemin were stored as 10 mM solutions at -20°C in *N,N*-dimethylsulfoxide (**DMSO**).

Bacterial Strains and Growth Conditions

Staphylococcus aureus strains 25923 and 6538 were obtained from the American Type Culture Collection.

Five clinical isolates of *S. aureus* strains SHY97-3906, -3923, -4085, -4320, and strain RFT-5 from bovine mastitis cases were kindly provided by the Laboratoire Provincial de Pathologie Animale of St-Hyacinthe and of Rock-Forest (Quebec, Canada), respectively. Nine β -lactam antibiotic resistant *S. aureus* strains (PC-1, NCTC 9789, 2076, 22260, ST79/741, 3804, RN9, FAR8, and FAR10) were from Vanderbilt University School of Medicine (Nashville, TN). None of these strains was a small colony variant. All culture media were from Difco Laboratories (Detroit, MI). Bacteria from frozen stock were streaked onto tryptic soy or brain infusion agar plates and then incubated for 16 to 20 h at 37°C . For most experiments, the strains were subcultured onto Mueller Hinton agar plates (**MHA**) or broth (**MHB**) for an additional 16 to 20 h. Iron-restricted conditions were obtained after the addition of 50 to 160 $\mu\text{g/ml}$ of ethylenediamine di-O-hydroxyphenylacetic acid (**EDDHA**; Sigma) or 0.2 mM of 2,2'-dipyridyl (Sigma). Iron-rich media were obtained by adding 5 μM FeCl_3 (Sigma). Aqueous solutions of the tested compounds were added by sterile filtration through a sterile filter assembly (pore size 0.2 μm , VWR Canlab, Mont-Royal, QC).

Growth Promotion Assay

This assay was performed in duplicate on all the *S. aureus* strains used in this study. Bacteria were tested for their ability to use different sources of iron by using a growth promotion test (Diarra et al., 1996). The plates were made iron deficient by supplementing MHA with 50 or 100 $\mu\text{g/ml}$ of EDDHA or 200 μM of 2,2'-dipyridyl (alone or with 1 mg/ml of lactoferrin), were inoculated with a sterile cotton swab dipped in a bacterial suspension in saline (approximately 10^8 cfu/ml). Disks (diameter, 6 mm) containing 0.04 μmol of DFO, 0.04 μmol of hemin, 200 μg of bovine hemoglobin, 200 μg of horse spleen ferritin or 1 mg of lactoferrin were placed on the surface of agar plates to allow growth promotion. Plates were incubated at 37°C in 5% of CO_2 for 20 h and then growth promotion zones were measured around the disks. Disks containing diluted DMSO were used as controls.

Siderophore Production Assay

Siderophore production was evaluated in all the *S. aureus* strains used in this study by a qualitative chromogenic assay using chrome azurol S (**CAS**; Sigma) in the culture medium (Neilands and Nakamura, 1991). This is a highly sensitive chemical method for the detection of siderophores, which is based on their affinity for Fe^{3+} and is therefore independent of their

chemical structure. When a strong chelator (i.e., siderophore) removes iron from the dye, its color turns from blue to orange. Agar plates were supplemented with 100 μ M 2,2'-dipyridyl in addition to CAS and were inoculated with overnight cultures. *Citrobacter diversus* SHY99-723-4 (Laboratoire de pathologie animale St-Hyacinthe) and *Streptococcus dysgalactiae* RFT-2 (Laboratoire de pathologie animale, Rock-Forrest) were used as positive and negative controls, respectively.

Growth Curves

Staphylococcus aureus strains ATCC 25923, SHY97-3906, and SHY97-4320 were used to evaluate growth rate in liquid media (Diarra et al., 1996). Briefly, 2 ml of overnight cultures in MHB was used to inoculate 50 ml of fresh MHB, MHB deferrated with 100 μ g/ml of EDDHA, MHB deferrated with EDDHA and supplemented with 25 μ M of either ferrichrome or DFO. All flasks were incubated at 37°C with agitation (150 rpm) for 8 h. Aliquots were removed every hour to determine the culture turbidity (optical density at 540 nm).

Hemin Binding Assay

The bovine hemin binding activity was evaluated by modification of the procedure described by Tai et al. (1997). *Staphylococcus aureus* strains ATCC 25923, SHY97-3906 SHY97-4320, and RFT-5 from overnight culture were washed and suspended in 0.01 M PBS at pH 7.5 containing 0.0027 M potassium chloride and 0.137 M sodium chloride to an optical density of 0.8 at 600 nm. For dose-dependent hemin binding assay, aliquots of this suspension were mixed with hemin at concentrations of 0, 10, 20, 40, and 60 μ g/ml. The effects of inoculum size were also evaluated by mixing 40 μ g/ml of hemin with bacterial suspension at an optical density of 0.8, 0.4, 0.3, and 0.1 at 600 nm. The reaction mixtures (4 ml) containing approximately 2.5×10^8 bacteria/ml were then incubated at 37°C with agitation (150 rpm) for 30 or 60 min. Aliquots of 1 ml were removed and centrifuged for 1 min at $14,000 \times g$ to pellet the cells, and the unbound hemin was determined by spectrophotometer in the supernatant at an optical density of 400 nm (Deneer and Potter, 1989). The amount of bound hemin was obtained by subtraction. For binding kinetic study, cells from overnight culture were suspended in PBS to an optical density of 1.5 at 600 nm and hemin was added to a final concentration of 40 μ g/ml. Aliquots were removed every 5 min to determine the unbound hemin.

Biotinylation of Lactoferrin

The biotinylation of lactoferrin was performed using a modified version of the method described by Rejman et al. (1994). Lactoferrin was suspended in PBS pH 7.5 at a final concentration of 1 mg/ml. N-Hydroxy-succinimide-biotin (**NHS-biotin**, Pierce, Rockford, IL) was dissolved in DMSO to yield a stock solution of 15.6 mg/ml. The NHS-biotin (250 μ g) was allowed to react with apo or iron-saturated lactoferrin solution for 2 h on ice with gentle agitation. The unreacted biotin was removed by three ultrafiltrations with a Centriflo membrane cone (Amicon Corporation, Danvers, MA) and stored at 4°C. Iron-saturated lactoferrin was obtained by dissolving lactoferrin in 0.1 M sodium citrate, 0.1 M sodium carbonate pH 8.6 buffer containing a 50-fold molar excess of FeCl_3 . The excess of reagents was removed by ultrafiltration with a Centriflo membrane cone.

Lactoferrin Binding Assay

Staphylococcus aureus strains ATCC 25923 and SHY97-3906 were used to perform the dot blot assay as described by Schryvers and Gonzalez (1989). Bacterial cells grown on iron-rich or iron-poor media were suspended in PBS to an optical density of 2 at 600 nm and then serially diluted in the same buffer to have optical densities of 2, 1, 0.5, 0.12, 0.06, and 0.03 at 600 nm. A volume of 10 μ l of each dilution was directly spotted onto nitrocellulose membranes (0.45 μ m Bio-Rad, Richmond, CA). Competition binding assays were performed by applying on bacterial spots, different concentrations of lactoferrin diluted in PBS. The membranes were blocked with 2% skim milk in Tris-saline buffer (**TSB**; 50 mM Tris, 150 mM NaCl, pH 8.0). All other incubation steps were followed by 3-min washes with TSB. Nitrocellulose membranes were incubated for 1 h with either NHS-biotin labeled with bovine apo- or iron-saturated lactoferrin. Reaction was revealed with streptavidin conjugated to alkaline phosphatase (**streptavidin-AP**) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate to-luidine substrate (**NBT/BCIP**, Boehringer, Montreal, Canada). Binding intensity was determined on Bio-Rad imaging densitometer model GS-670 using Molecular Analyst software (Bio-Rad Laboratories, Richmond, CA).

SDS-PAGE and Ligand Blot Analysis

Whole cells of *S. aureus* strains ATCC 25923, SHY97-3906, and SHY97-4320 from iron-rich or iron-restricted media were suspended in electrophoresis sample buffer containing 2% SDS and 5% 2-mercapto-

ethanol to a final concentration of 0.1 g/ml. The samples were heated at 100°C for 5 min before being loaded for electrophoresis on a discontinuous 0.1% SDS-12% polyacrylamide gel system (Laemmli, 1970). Protein profile was visualized by staining with Coomassie brilliant blue. Electrophoretic transfer of SDS-polyacrylamide gel separated proteins to nitrocellulose membranes and probing with biotin-lactoferrin were performed essentially as described by Towbin et al. (1979). Nonspecific binding sites were blocked by incubating the membranes for 1 h at room temperature in TSB (pH 8.0) containing 3% gelatine. Membrane was then incubated for 1 h with biotinylated lactoferrin. Reaction was revealed with streptavidin-AP and NBT/BCIP substrate.

Statistics

Each assay was performed at least in two duplicate separate experiments. Data of binding intensity and of growth promotion in disc diffusion assay were analyzed with the GLM procedure of SAS (1985). Effects of siderophores on growth rate in liquid media and binding of hemin over time were analyzed as a repeated measurement design using the same procedure. Least squares means and standard deviations are presented in Figures 1 and 2.

RESULTS

Growth Promotion by Siderophores

The addition of the iron chelator EDDHA in the nutrient media caused a dose dependent growth inhibition of bacteria. However, complete growth inhibition of *S. aureus* was not achieved despite the use of high concentration (160 µg/ml) of EDDHA. The ability of ferrichrome, DFO, bovine hemin, and hemoglobin to reverse the growth inhibition by EDDHA was evaluated using a growth promotion assay (Figure 1A). Results indicate that all *S. aureus* strains tested can obtain iron for growth from both hydroxamate siderophores (ferrichrome and DFO) as well as from the heme proteins (hemin and hemoglobin) in cultures lacking other iron sources. Desferrioxamine and ferrichrome were the best promoting agents ($P < 0.001$). Sizes of the growth promotion zones were reduced slightly by EDDHA ($P < 0.01$).

Growth promotion assay was also evaluated in the presence of the iron chelator 2,2'-dipyridyl alone or in combination with lactoferrin (Table 1). In media containing dipyridyl alone, basal growth was important and no growth promotion zone was detected. However, growth was inhibited around the paper disc impregnated with ferritin in 7 of the 13 tested strains.

Figure 1. Growth promotion of *Staphylococcus aureus*. **A**, Average promotion zone sizes from 16 strains obtained in disk diffusion assay, obtaining with 0.04 µmol desferrioxamine B, ferrichrome, and hemin, or with 200 µg hemoglobin on MHA supplemented with 50, 100, or 160 µg/ml EDDHA. **B**, Average growth rate of *S. aureus* in MHB (□), MHB deferrated with 100 µg/ml of EDDHA (○), MHB deferrated with EDDHA and supplemented with 25 µM of either ferrichrome (×) or desferrioxamine B (△).

When lactoferrin and dipyridyl were combined, greater inhibitory zones were observed with ferritin in susceptible strains, whereas the others strains showed growth promotion zone with ferritin. A growth promotion zone was also seen around the discs loaded with hemoglobin, hemin, and DFO in the presence of dipyridyl and lactoferrin. A small growth inhibition zone was observed with discs loaded with 1 mg of lactoferrin (Table 1).

Growth Curves and Detection of Siderophore Production

Staphylococcus aureus strains ATCC 25923, SHY97-3906, and SHY97-4320 were used to evaluate the growth rate in liquid culture deferrated by the addition of 100 µg/ml of EDDHA and supplemented with ferrichrome or DFO. The growth rate patterns

Table 1. Growth promotion of *Staphylococcus aureus* strains in disc diffusion assay achieved with disc containing either 0.04 μ mol of desferrioxamine B (DFO), 0.04 μ mol of hemin (He), 200 μ g of bovine hemoglobin (Hb), 200 μ g of horse spleen ferritin (Fer), or 1 mg of lactoferrin (Lf) on MHA supplemented with 200 μ M 2,2'-dipyridyl alone or in combination with 1 mg/ml of bovine lactoferrin.

Strain	Average diameter (mm) of zone of growth promotion									
	2,2'-Dipyridyl					2,2'-Dipyridyl + lactoferrin				
	Hb	He	DFO	Fer	Lf	Hb	He	DFO	Fer	
SHY97-3906	0	0	0	-19	0	14.5	10	24	-27.5	
SHY97-3923	0	0	0	-17	nd	14	10	24	-26	
SHY97-4320	0	0	0	0	-8	14.5	11	28	12	
ATCC 25923	0	0	0	0	0	14	10	26.5	11.5	
PC-1	0	0	0	-19	0	13	12	28.5	-29	
NCTC 9789	0	0	0	0	nd	14	11.5	27	13	
2076	0	0	0	-17	nd	14.5	12	27	-25.5	
22260	0	0	0	-18.5	0	13.5	11	26	-24	
ST79/741	0	0	0	0	nd	14.5	11	26	14.5	
3084	0	0	0	0	-7	14.5	10.5	28	13	
RN9	0	0	0	-22	nd	14.5	11	29	-27.5	
FAR8	0	0	0	0	-8	15	12	27.5	13	
RFA10	0	0	0	0	nd	15	12	28	12.5	

nd = Not determined.

were similar for all three strains tested. As shown in Figure 1B, the addition of 100 μ g/ml of EDDHA to the MHB reduced ($P < 0.001$) the growth rate of *S. aureus*. This growth inhibition was largely reversed by the presence of 25 μ M of ferrichrome or DFO ($P < 0.001$). A significant difference was not observed between DFO and ferrichrome ($P > 0.05$).

Chromogenic blue CAS agar plates were used to determine whether the tested *S. aureus* strains produce siderophores in response to iron stress. All the strains of *S. aureus* exhibited a distinct yellow halo around the colonies indicative of the production of iron chelators by the strains. In CAS test without dipyr-dyl, no iron chelator activity was observed.

Hemin-Binding

As all the tested staphylococcal strains were able to use hemin as an iron source for growth, we investigated the binding of this protein to the cells of *S. aureus* strains SHY97-4320, ATCC 25923, and RFT-5. The hemin binding activity for *S. aureus* SHY97-4320, which was isolated from a clinical bovine mastitis, is presented in Figure 2. The amount of hemin bound increased linearly with the hemin concentration ($P < 0.001$) in the media up to 40 μ g/ml (Figure 2A). Binding of hemin increased proportionally with the number of bacterial cells present in the assay ($P < 0.001$; Figure 2B). We have also studied the binding kinetic of hemin in strain SHY97-4320 after growth in iron-rich and iron-poor media. Results indicated that hemin binding activity was reduced ($P < 0.001$) by previous growth under the iron-restricted condition

brought by EDDHA (Figure 2C). Lactoferrin did not significantly affect hemin binding. Strains ATCC 25923 and RFT-5 also bind hemin, and the binding activity was dependent on the dose of hemin, the inoculum size, and the incubation time (data not shown).

Binding of Lactoferrin to Bacterial Cells and Identification of Lactoferrin-Binding Proteins

Lactoferrin-binding activity was evaluated in intact cells of *S. aureus* strains ATCC 25923 and SHY97-3906. Both strains of bacteria, whether grown in iron-rich or iron-poor MHB, can bind both the apo-form and iron-saturated forms of biotin-labeled bovine lactoferrin (data not shown). The protein profiles of whole cells of *S. aureus* strains ATCC 25923, SHY97-3906, and SHY97-4320 obtained on SDS-polyacrylamide gels after electrophoresis were examined after having been grown in MHB and MHB supplemented with 100 μ g/ml of EDDHA. The biotinylated-lactoferrin recognized proteins of molecular weight ranging from 32 to 92 kDa. Pattern of lactoferrin binding was not affected consistently by iron restriction.

DISCUSSION

Staphylococcus aureus is able to grow in the presence of extremely low (0.04 μ M) iron concentrations (Trivier and Courcol, 1996). Accordingly, we showed the capability of this bacteria to growth in the presence of a high dose (160 μ g/ml) of the iron chelator EDDHA. The production of siderophore demonstrated in this study and by other authors (Haag et al., 1994) could be

Figure 2. Binding of hemin to *Staphylococcus aureus* SHY97-4320 growth in Mueller Hinton agar broth. (A) Dose and (B) inoculum dependant binding activity after incubation of cells at 37°C for 30 (□) or 60 min (■). (C) Kinetic of hemin utilization after growth in the presence of ethylenediamine di-O-hydroxyphenylacetic acid (EDDHA) (100 μg/ml), 1 mg/ml of bovine lactoferrin (LF) alone or in combination.

part of the adaptation of *S. aureus* to iron starvation. Moreover, our results with the ferrichrome and DFO indicated that all tested strains of *S. aureus* were also able to use exogenous hydroxamate siderophores as a growth-promoting agent under iron-limited condi-

tions. Ferrichrome is a cyclic hexapeptide produced by many fungal species including *Ustilago sphaerogena*, some *Aspergillus*, and all *Penicillium* species, whereas DFO belongs to a large family of siderophores (ferrioxamines) excreted by the *Actinomycetes* (Martinez et al., 1990). The use of exogenous hydroxamate siderophores by *S. aureus* could be important in situations of coexistence with siderophore-producing microorganisms in their habitat niches. The presence of these fungi in the mammary gland is unlikely but some are present in cattle feeds. Siderophores are small molecules; therefore, it might be possible that some of them are absorbed and transferred into milk. In *E. coli* and *Salmonella typhimurium* strains, the use of exogenous siderophores was described to be due to the presence of outer membrane receptors for siderophores that they do not produce (Martinez et al., 1990). Recently, a membrane permease involved in iron-hydroxamate transport in *S. aureus* was identified (Sebulsky et al., 2000).

Iron is an essential nutrient for nearly all forms of life, but its insolubility and reactivity lead to problems of poor availability and toxicity. To provide sufficient quantities of iron while maintaining it in a nontoxic state, storage proteins, known as ferritins, are used. These proteins ($M_r \sim 500,000$) present in animals, plants (phytoferritins), and bacteria (bacterioferritin) can accommodate up to 4500 iron atoms (Ratledge and Dover, 2000). Stored iron that can be used for metabolism cannot participate in free radical-generating reactions (Ghio et al., 1998). During mammary infection with coagulase-negative staphylococci, iron concentrations in milk increase (Burriel and Heys, 1997). That increase might be due to the death of epithelial cells leading to the release of ferritin into milk. Bacteria can obtain iron from ferritin either by siderophore or by direct contact (Ratledge and Dover, 2000). Although all the *S. aureus* strains tested were able to produce siderophores, fewer than half of them used ferritin as an iron source under iron-restricted conditions. Moreover, this protein in both iron-rich and iron-poor conditions inhibited the growth of strains unable to use it as an iron source. The difference in the ability of *S. aureus* to use ferritin suggests that siderophores alone are not sufficient to acquire iron from ferritin. It can be speculated that the release of iron from ferritin depends on the production by *S. aureus* of an extracellular protease, which is able to cleave ferritin.

Within host cells, heme and heme proteins are important sources of iron. In extracellular space, heme bound to protein such as albumin, hemopexin, and haptoglobin are also important iron sources for bacteria. In this study, we observed that *S. aureus* effi-

ciently bind to hemin. It can be speculated that *S. aureus* possess specific hemin binding sites as a saturation of hemin binding activity is observed with concentration of hemin in the medium over 40 µg/ml. Other pathogenic bacteria, such as *Streptococcus pneumoniae* and *Actinobacillus pleuropneumoniae*, have been reported to be able to use hemin and hemo-globin for growth under low-iron conditions via a heme transport system (Braun et al., 1998; Tai et al., 1997; Deneer and Potter, 1989). The use of hemin and hemo-globin by *S. aureus* can provide advantage to bacteria during mammary infection as blood proteins "leak" into milk during inflammation. Hemin-binding activity was not increased after growth of staphylococcal cells in iron-poor media obtained by addition EDDHA and/or lactoferrin. This observation is different from those made in other bacteria. Indeed, the use of heme proteins and transferrin has been shown to be negatively regulated by iron in *E. coli*, *Serratia marcescens*, and *Pseudomonas aeruginosa* (Braun and Killman, 1999; Lim et al., 1998). It remains to be established whether the staphylococcal heme protein acquisition system is different to those of *E. coli* and other bacteria.

Lactoferrin did not promote growth of any of the strains of *S. aureus* tested, indicating that none of them were able to use this iron chelator under our conditions. Moreover, a small growth inhibition zone was detected. Lactoferrin, in its iron-limited form, has been shown to inhibit the growth of many pathogenic bacteria by depriving them of essential iron for their multiplication. Our results showed that supplementation of iron-restricted media with lactoferrin did not prevent the use of DFO, ferrichrome, and heme proteins by *S. aureus*. Nevertheless, lactoferrin was able to specifically bind to the cells of all *S. aureus* strains tested. Biotinylated bovine lactoferrin bound to proteins with molecular masses ranging from 32 to 92 kDa. Naidu et al. (1991) demonstrated that bovine lactoferrin was able to bind to more than 85% of *S. aureus* strains. Those authors reported two lactoferrin-binding proteins with molecular weights of approximately 67 and 92 kDa in *S. aureus* strain SA-340. A 450-kDa *S. aureus* human lactoferrin-binding protein complex that consists of two protein subunit components of 62 and 67 kDa upon exposure to reducing conditions was also reported by Naidu et al. (1992). Lactoferrin has also been reported to interact with gram-negative bacterial cell surface components such as porins (Erdei et al., 1994) and lipid A of LPS (Appel-melk et al., 1994). Griffiths (1987) reported that the direct contact between the cell surface and lactoferrin could inhibit glucose uptake and metabolism and synthesis of macromolecules. The role in iron acquisition

of the staphylococcal lactoferrin-binding proteins detected in the present study and that of those published by others remains to be determined.

In conclusion, these results indicate that *S. aureus* has multiple iron uptake systems, which may contribute to its ability to live in environments with variable concentrations of iron.

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